# Uptake by rat liver and intracellular fate of plasmid DNA complexed with poly-L-lysine or poly-D-lysine

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**Abstract Efficiency of transfection is probably dependent on the rate of intracellular degradation of plasmid DNA. When a non-viral vector is used, it is not known to what extent the plasmid DNA catabolism is subordinated to the catabolism of the vector. In the work reported here, the problem was approached by following the intracellular fate in rat liver, of plasmid [$^{35}$S]DNA complexed with a cationic peptide poly-L-lysine that can be hydrolyzed by cellular peptidases or with its stereoisomer, poly-D-lysine, that cannot be split by these enzymes. Complexes of DNA with poly-L-lysine and poly-D-lysine are taken up to the same extent by the liver, mainly by Kupffer cells, but the intracellular degradation of nucleic acid molecules is markedly quicker when poly-L-lysine is injected. The association of DNA with the polycations inhibits DNA hydrolysis in vitro by purified lysosomes but similarly for poly-L-lysine and poly-D-lysine. The intracellular journey followed by [$^{35}$S]DNA complexed with poly-L- or poly-D-lysine was investigated using differential and isopycnic centrifugation. Results indicate that [$^{35}$S]DNA is transferred more slowly to lysosomes, the main site of intracellular degradation of endocytosed macromolecules, when it is given as a complex with poly-D-lysine than with poly-L-lysine. They suggest that the digestion of the vector in a prelysosomal compartment is required to allow endocytosed plasmid DNA to rapidly reach lysosomes. Such a phenomenon could explain why injected plasmid DNA is more stable in vivo when it is associated with poly-D-lysine.**



*Key words:* Lysosome; Cationic peptide; Transfection; Non-viral vector

## 1. Introduction

Efficiency of transfection is probably dependent on the rate of intracellular degradation of plasmid DNA. When nucleic acid molecules are complexed with a vector such as a cationic peptide (polylysine, polyarginine) or a cationic lipid, they are taken up by endocytosis [1] and therefore are mostly degraded in lysosomes, the main site of endocytosed macromolecule hydrolysis [2]. Two factors will chiefly influence the rate of plasmid DNA degradation: the rate with which nucleic acid molecules are delivered to lysosomes after having been picked up by the cells and the susceptibility of these molecules to lysosomal nucleases. Cationic compounds could affect these factors by making nucleic acids more resistant to nucleases, as has been shown in vitro [3], and by delaying their transfer to lysosomes [4,5]. How the type of cationic compound influences the intracellular degradation of plasmid DNA is poorly documented. Particularly, it is not known to what extent the plasmid DNA catabolism is dependent on the catabolism of the vector when a non-viral vector is used. The problem was approached in the work presented here, by following the intracellular fate in rat liver of plasmid [$^{35}$S]DNA complexed with a cationic peptide poly-L-lysine that can be hydrolyzed by cellular peptidases, or with its stereoisomer poly-D-lysine that cannot been split by these enzymes. Our results show that in vivo, the rate of DNA degradation is markedly lower when the plasmid was complexed with poly-D-lysine than when it was associated with poly-L-lysine. Analysis of intracellular distribution of radioactivity by centrifugation indicates that DNA is transferred more slowly to lysosomes when it is given as a complex with poly-D-lysine than with poly-L-lysine. Such a phenomenon could explain why plasmid DNA is more stable in vivo when it is associated with poly-D-lysine.

## 2. Materials and methods

Experiments were performed with male Wistar rats weighing 300–350 g. Labeled DNA was obtained by nick translation of pGL3 control vector (Promega, Madison, USA) with [$^{35}$S]dATP. Labeling was performed with a kit from Amersham (Buckinghamshire, UK), according to the protocol provided by the manufacturer, except that a 10-fold excess of template was used and that the reaction was supplemented with 10 units of DNA polymerase I (Boehringer, Mannheim, Germany). Such a procedure makes it possible to obtain a labeled full length DNA as shown by agarose gel electrophoresis. For injection, 1 μg of [$^{35}$S]DNA was mixed with 5 μg of poly-L- or poly-D-lysine (75–150 kDa, Sigma) in a volume of 25 μl; after 10 min, 0.6 ml of 0.15 M NaCl was added. We found that the sedimentation profile after centrifugation in 0.15 M NaCl is the same for the two complexes obtained in these conditions. We also checked that complexes with poly-L-lysine are totally dissociated after treatment with trypsin whereas complexes with poly-D-lysine are stable in the presence of the proteolytic enzyme.

Rats were injected intravenously and killed at various times after injection. The liver was perfused with cold 0.15 M NaCl, removed and homogenized in ice-cold 0.25 M sucrose. The homogenate was fractionated by differential centrifugation as described by de Duve et al. [6], giving a nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a soluble fraction S. Isopycnic centrifugation of the total mitochondrial fraction (M+L) was performed according to Beaufay et al. [7], at 240 000 × *g* in a VTI65 Beckman rotor for 180 min. The sucrose gradient extended from 1.09 to 1.35 g/ml. Arylsulfatase was measured by the method of Bowers et al. [8] and proteins according to Lowry et al. [9]. Degradation of [$^{35}$S]DNA after its uptake was assessed by measuring the acid-soluble radioactivity in 5% perchloric acid. Purification of rat liver lysosomes was achieved by the method of Wattiaux et al. [10]. Separation of liver cells (hepatocytes, endothelial cells and Kupffer cells) was performed by the method of Seglen [11].

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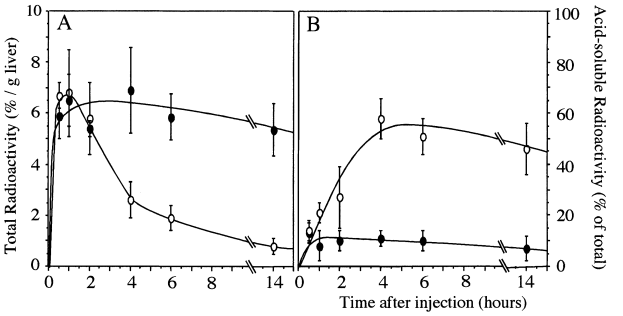


Fig. 1. Uptake of [$^{35}$S]DNA by rat liver. Radioactivity was measured in homogenates of rat liver at increasing times after injection of [$^{35}$S]DNA associated with poly-L-lysine (open circle) or poly-D-lysine (closed circle). A: Total radioactivity. The values are given as percentages of the injected dose/g liver. B: Acid-soluble radioactivity. The values are given as percentages of the total radioactivity. Means of at least three animals with S.D. are presented.

## 3. Results

### 3.1. Uptake of [$^{35}$S]DNA by rat liver

As illustrated in Fig. 1A, the complexes of DNA with poly-L-lysine and poly-D-lysine are taken up by the liver to the same extent, the maximal amount being reached after 30–60 min. Radioactivity originating from poly-D-lysine remains constant and mostly acid-precipitable (Fig. 1B) for many hours. In contrast, 60 min after poly-L-lysine injection, the liver radioactivity decreases and becomes more and more acid-soluble. Thus, apparently, the rate of plasmid DNA degradation is higher when the nucleic acid molecules are taken up as a complex with poly-L-lysine than when they are associated with poly-D-lysine.

### 3.2. Degradation of [$^{35}$S]DNA by purified lysosomes

The main intracellular destination of an endocytosed macromolecule is the lysosomes where these compounds are subjected to degradation by hydrolases present in these organelles

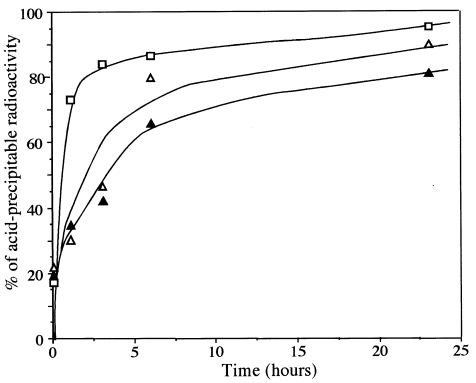


Fig. 2. Degradation of [$^{35}$S]DNA by purified lysosomes. 1 μg of [$^{35}$S]DNA was incubated for increasing times at 37°C in a medium containing 0.05 M acetate buffer pH 5 and purified rat liver lysosomes (25 μg protein), in a volume of 1 ml. The reaction was stopped by addition of the same volume of 10% perchloric acid. The precipitate was discarded by centrifugation and the radioactivity measured in the supernatant. Acid-soluble radioactivity is given as a percentage of the total radioactivity present in the incubation medium. Open square: naked [$^{35}$S]DNA; open triangle: [$^{35}$S]DNA complexed with poly-L-lysine; closed triangle: [$^{35}$S]DNA complexed with poly-D-lysine.

[2]. We have measured the rate of hydrolysis by purified rat liver lysosomes of plasmid [$^{35}$S]DNA, naked or complexed with poly-L- or poly-D-lysine (Fig. 2). The association of DNA with the polypeptides inhibits DNA hydrolysis by lysosomal nucleases but similarly for the two polymers. This indicates that the difference of DNA stability in vivo, depending on the fact that poly-L- or poly-D-lysine is used, probably does not originate from a difference of resistance to hydrolysis of the molecule by lysosomal nucleases.

### 3.3. Intracellular journey of [$^{35}$S]DNA

The intracellular journey followed by plasmid [$^{35}$S]DNA complexed with poly-L- or poly-D-lysine was investigated by centrifugation methods. First, liver homogenates from rats were analyzed by differential centrifugation according to de Duve et al. [6], the animals being killed 1, 4 or 14 h after injection. Results are presented in Fig. 3 according to the method of de Duve et al. [6], shaded areas indicate the proportion of acid-soluble radioactivity. One hour after poly-L-lysine injection, the largest part of radioactivity, mostly acid-precipitable, is recovered in the heavy mitochondrial fraction M. Later, the distribution profile of radioactivity becomes similar to that of lysosomal enzymes (exemplified by arylsulfatase distribution) except that a relatively high amount of radioactivity (totally acid-soluble) is present in the unsedimentable S fraction. A significant proportion of radioactivity found in the mitochondrial fractions M and L is acid-soluble. One hour after poly-D-lysine injection, the radioactivity distribution does not differ from that observed after poly-L-lysine

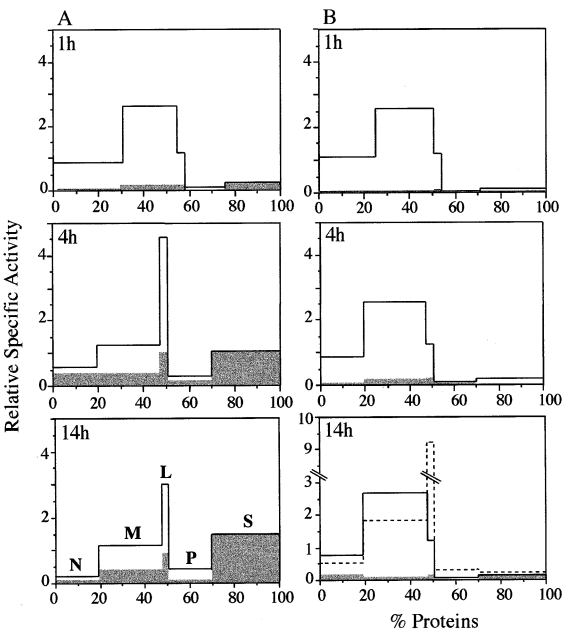


Fig. 3. Distribution of radioactivity after differential centrifugation. The radioactivity distributions were obtained with livers of rats killed at increasing times after injection of [$^{35}$S]DNA associated with poly-L-lysine (A) or poly-D-lysine (B). Ordinate: relative specific radioactivity of fractions (percentage of total recovered radioactivity/percentage of total recovered proteins); abscissa: relative protein content of fractions (cumulatively from left to right). N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S soluble fraction. Shaded areas represent the percentage of acid-soluble radioactivity found in the fractions. In broken line, a representative distribution of arylsulfatase.

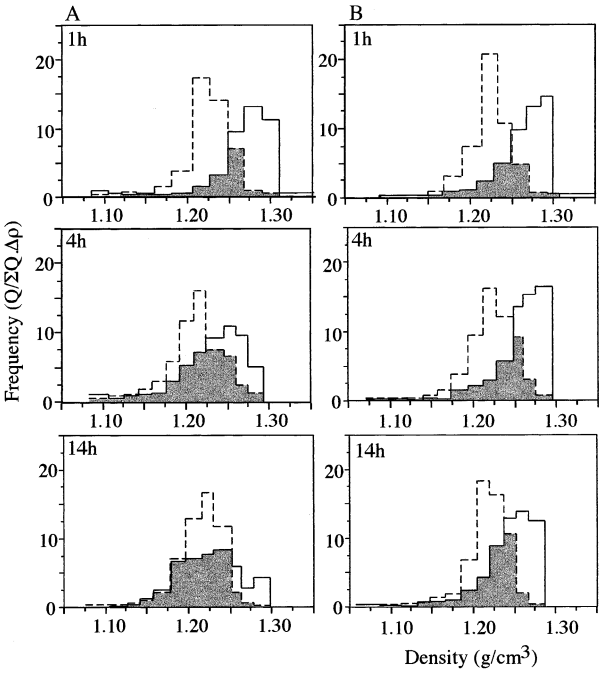


Fig. 4. Density distribution histograms of radioactivity (continuous line) and arylsulfatase (broken line) after isopycnic centrifugation of a total mitochondrial fraction (M+L) in a sucrose gradient. The particle preparations were isolated at increasing times after injection of [$^{35}$S]DNA associated with poly-L-lysine (A) or poly-D-lysine (B). Centrifugations were performed at 240 000 × $g$ in a VTI 65 Beckman rotor for 180 min. The sucrose density gradient extended from 1.09 to 1.30 g/cm$^3$ density. Ordinate: frequency $Q/\Sigma Q.\Delta\rho$ where $Q$ represents the activity found in the fraction, $\Sigma Q$ the total activity recovered in the sum of the fractions and $\Delta\rho$ the increment of density from top to bottom of the fraction.

injection but remains unchanged even after 14 h; moreover, at any time, radioactivity is almost totally acid-precipitable.

To more clearly characterize the radioactivity bearing structures that are mainly present in the M and L fractions, granule preparations corresponding to the sum of these fractions were analyzed by isopycnic centrifugation in a sucrose gradient. Results are given in Fig. 4. One hour after poly-L-lysine or poly-D-lysine injection, radioactivity is located in organelles the distribution of which exhibits only a limited overlap (shaded areas) with that of arylsulfatase, marker of lysosomes. These structures are mainly recovered in high density regions of the gradient. Four hours after injection of poly-L-lysine, the radioactivity distribution curve is shifted towards lower density regions and markedly overlaps the distribution curve of the lysosomal enzyme; this phenomenon is still more evident after 14 h. When poly-D-lysine was injected, radioactivity remains located to a large extent in high density regions of the gradient, even after 14 h.

### 3.4. Effect of Triton WR 1339

Differential and isopycnic centrifugation results strongly suggest that the transfer of radioactivity to lysosomes is more rapid when [$^{35}$S]DNA is complexed with poly-L-lysine. An interesting method to assess the lysosomal location of a compound is to specifically change the density of lysosomes by injecting the animal with a substance that accumulates in these organelles because it cannot be digested by lysosomal hydrolases. As a result, the distribution profile of lysosomal enzyme in a density gradient is shifted towards lower or higher densities [11]. If a substance (an endocytosed molecule for example), is associated with lysosomes, its distribution profile will be similarly affected [12]. Triton WR 1339, a non-ionic detergent of low density, is particularly suitable for this purpose: it is endocytosed by the liver and, being resistant to digestion by lysosomal hydrolases, it accumulates in these organelles and decreases their density [13]. Fig. 5 illustrates the effect of Triton WR 1339 treatment on the distribution of radioactivity at increasing times after poly-L-lysine or poly-D-lysine injection and on the distribution of arylsulfatase. As expected, lysosomal hydrolase distribution curve is strikingly shifted towards low density regions. One hour after injection of poly-L-lysine or poly-D-lysine, the major part of radioactivity remains well separated from arylsulfatase, as is also seen in normal rats (see Fig. 4), and is slightly affected by Triton WR 1339 treatment. Four hours after poly-L-lysine injection, a large proportion of radioactivity is recovered in low density zones like arylsulfatase and has been subjected to the same shift of distribution as the lysosomal enzyme by Triton WR 1339. The distribution shift caused by Triton WR 1339 is less pronounced for radioactivity originating from poly-D-lysine indicating a slower transfer of labeled molecules to lysosomes.

### 3.5. Distribution of [$^{35}$S]DNA in liver cells

Three main cell types are present in the liver: hepatocytes, endothelial cells and Kupffer cells. We investigated whether the cellular location of plasmid DNA taken up by the liver depended on the cationic compound with which it was associated. As illustrated in Fig. 6, most of the radioactivity found in the liver 1 h after poly-L-lysine or poly-D-lysine injection is

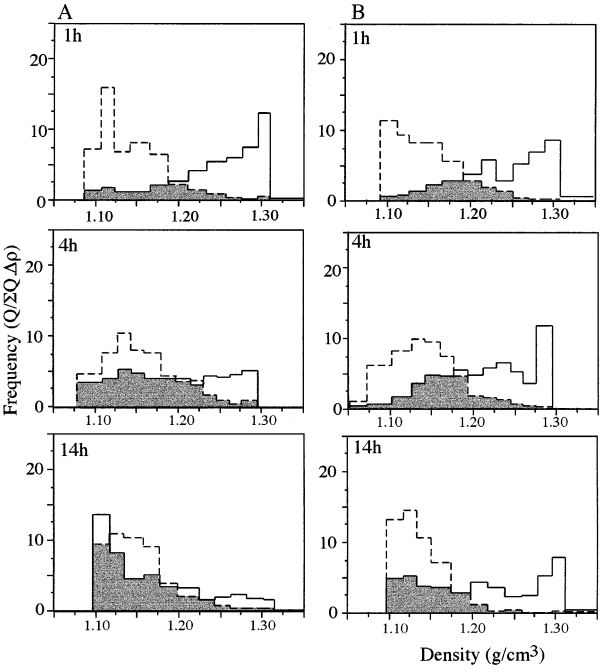


Fig. 5. Density distribution histograms of radioactivity (continuous line) and arylsulfatase (broken line) after isopycnic centrifugation of a total mitochondrial fraction (M+L) in a sucrose gradient. Effect of Triton WR 1339. Experiments were performed as described in the legend of Fig. 4 with rats intravenously injected with Triton WR 1339 (170 mg in 1 cm$^3$ of saline) 4 days before injection of [$^{35}$S]DNA associated with poly-L-lysine (A) or poly-D-lysine (B).

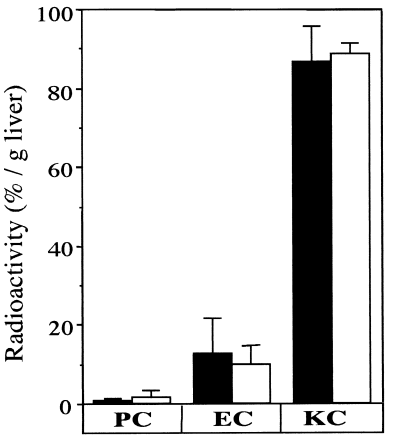


Fig. 6. Distribution of radioactivity in rat liver cells. The radioactivity distributions were obtained with livers of rats killed 1 h after injection of [$^{35}$S]DNA associated with poly-L-lysine (open bar) or poly-D-lysine (closed bar). Values are given as percentages of total liver radioactivity. Means of at least three animals with S.D. are presented. PC, parenchymal cells; EC, endothelial cells; KC, Kupffer cells.

located in non-parenchymal cells, to a large extent in Kupffer cells. No significant differences were observed between the two complexes. Hence, the differences we observed between the fates of poly-L-lysine and poly-D-lysine do not arise from a difference of cellular location in the liver.

## 4. Discussion

Our results show that the intracellular degradation of a plasmid DNA injected as a complex with a cationic vector poly-L-lysine or poly-D-lysine is markedly more rapid when the vector is poly-L-lysine. The two polycations have the same molecular weight and the same charge. They give rise to complexes with plasmid DNA exhibiting the same size, as shown by their sedimentation properties in a centrifugal field, that are taken up to the same extent by sinusoidal cells of the liver. In fact, the main and probably the only difference between the two molecules that we have to consider here is that poly-L-lysine is metabolizable while poly-D-lysine is not. How such a difference can influence the intracellular degradation of plasmid DNA was investigated here.

The lysosomes are the main site of hydrolysis of plasmid DNA when it is endocytosed as a complex with a cationic vector. As has been shown previously, cationic lipids delay the transfer of plasmid DNA to lysosomes [4,5]. The same is true for polylysine. Indeed, according to our centrifugation results, 1 h after injection of the labeled complexes, most of the radioactivity originating from poly-L-lysine or poly-D-lysine is located in non-lysosomal structures and is acid-precipitable, whereas at this time it is already present in lysosomes when naked [$^{35}$S]DNA was injected [4]. However, later it is clear that transfer to lysosomes takes place but at a rate that is lower when the polycation is poly-D-lysine. Four hours after poly-L-lysine injection, most of the radioactivity recovered in the mitochondrial fractions is distributed like lysosomes in differential and isopycnic centrifugation. Moreover, the fact that a large amount of radioactivity is present in the non-sedimentable fraction of the homogenate and is acid-soluble indicates that part of the plasmid DNA has already been hydrolyzed, probably by lysosomal nucleases, the digestion products having diffused in the cytosol. Radioactivity distributions observed after poly-D-lysine injection indicate that a longer time is required to clearly identify the association of a significant proportion of the radioactivity with lysosomes. One can roughly estimate the percentage of radioactivity present in lysosomes by measuring its distribution area that overlaps the distribution curve of arylsulfatase. Such calculations show that 4 h after injection of the complexes 70–80% of radioactivity sedimenting in the mitochondrial fractions are located in lysosomes when the vector is poly-L-lysine and about 45% when the vector is poly-D-lysine. It is to be noted that results are the same whether the rats are injected with Triton WR 1339 or not.

To explain why cationic lipids delay the transfer of plasmid DNA to lysosomes, we have proposed that, due to their size, DNA-cationic lipid complexes are taken up mostly by a process similar to phagocytosis [5] and that degradation of the complexes would be required to allow phagosomes to hand over their content to lysosomes [5], the delivery of phagocytic particles to lysosomes depending on their size [14]. The same hypothesis can be proposed to explain that polylysine delays the transfer of DNA to lysosomes.

Why is there a difference between poly-L- and poly-D-lysine? We think that it is because poly-L-lysine is degradable and poly-D-lysine is not. The presence of cathepsins in endosomes and the possible fusion of these organelles with phagosomes have been described [15–17]. Therefore, after internalization, DNA-polylysine complexes can be exposed to the peptidolytic action of these enzymes which are efficient on poly-L-lysine but not on poly-D-lysine. Hydrolysis of poly-L-lysine would dissociate the complexes, reducing their size and even releasing free DNA inside a prelysosomal compartment. As a result the transfer of nucleic acid molecules to lysosomes would take place but with a certain delay. Another consequence of poly-L-lysine hydrolysis in a prelysosomal compartment is that plasmid DNA released from the complex would be more easily digested by nucleases when it arrives in lysosomes. Such a process would not be possible with poly-D-lysine, which cannot be split by cathepsins.

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